

Differences Between HA Receptor-Binding Sites of Avian Influenza Viruses Isolated from Laridae and Anatidae

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Received 14 April 2002

SUMMARY. A comparative study of the hemagglutinin (HA) receptor binding site (RBS) of a number of H13 influenza viruses isolated from *Laridae* family of birds (gulls) and other influenza viruses obtained from the *Anatidae* family (ducks) was conducted. The affinity of all viruses to alpha *N*-acetylneuraminic acid (Neu5Ac α), 3'-sialyllactose (3'SL), and sialylglycopolymers bearing 3'-sialyl(*N*-acetylglucosamine) (3'SLN-PAA), [Neu5Ac α (2-3)Gal β (1-4)][-Fuc α (1-3)]GlcNAc β (SLe^x-PAA), and [Neu5Ac α (2-3)Gal β (1-3)][-Fuc α (1-4)]GlcNAc β (SLe^a-PAA), was determined. The last three polymer glycoconjugates were synthesized for determining the contribution of carbohydrate chains after the galactose link to the binding with the receptor. The difference in affinity between 3'SL and Neu5Ac α in all studied H13 viruses is small, which indicates a less significant role of the galactose moiety in the binding to the receptor. The results of virus binding with polymer sialylglycoconjugates indicates that the method of linking, the third monosaccharide moiety, and the presence of an extra fucose substitute in this moiety may influence the binding considerably. For viruses isolated from ducks, the suitable polymer is SLe^a-PAA (i.e., a 1-3 linkage between galactose and glucosamine is optimal). This finding is in accord with the data that H13 viruses isolated from the gulls differ based on their ability to interact with polymer sialylglycoconjugates. The affinity to all three polymers is uniform, and the presence of GlcNAc-linked fucose does not prevent the binding. A comparative analysis of six sequenced HA H13 viruses and other subtype viruses showed presence of substantial differences in the composition of amino acids of this region in H13 viruses.

RESUMEN. Diferencias entre los sitios de interacción de los receptores de la hemagglutina de los virus de influenza aislados a partir de aves de las especies laridae y anatidae.

Este es un estudio comparativo de los sitios de interacción de los receptores de la hemagglutina de varios aislados de virus de influenza del tipo H13 obtenidos a partir de aves de la familia *Laridae* (gaviotas) y otros virus de influenza obtenidos de aves de la familia *Anatidae* (patos). Se determinó la afinidad de todos los virus al ácido alfa *N*-neuramínico (Neu5Ac α), a la 3' sialolactosa (3'SL) y a los sialilglicopolímeros que contienen 3'-sialil(*N*-acetil lactosamina) (3'SLN-PAA), [Neu5Ac α (2-3)Gal β (1-4)][-Fuc α (1-3)]GlcNAc α (SLe^x-PAA) y [Neu5Ac α (2-3)Gal β (1-3)][-Fuc α (1-4)]GlcNAc α (SLe^a-PAA). Los últimos tres polímeros glucoconjugados mencionados fueron sintetizados con el fin de determinar la contribución de las cadenas de azúcares añadidas después de la galactosa en relación con la unión al receptor. La diferencia en afinidad entre el 3' SL y el Neu5Ac α en todos los virus del tipo H13 estudiados fue pequeña, lo que indica que la galactosa juega un papel menos significativo en la unión al receptor. Los resultados de la interacción de los virus con los polímeros sialilglucoconjugados indican que la forma química con un tercer residuo de monosacárido y la presencia de una sustitución extra de

This proceedings manuscript documents a poster presentation given in the Session on Pathology and Pathogenesis at the Fifth International Symposium on Avian Influenza, April 14–17, 2002, at The University of Georgia, Athens, GA 30602.

fucosa en este residuo puede influenciar la unión al receptor de una manera significativa. Para los virus aislados de patos el polímero mas adecuado fue el SLe^a-PAA, o sea una unión 1-3 entre la galactosa y la glucosamina es la forma química óptima. Este hallazgo concuerda con los datos obtenidos en estudios con virus del tipo H13 aislados a partir de gaviotas, los cuales difieren en su capacidad para interactuar con polímeros sialilglucoconjugados. La afinidad para los tres polímeros es uniforme y la presencia de un residuo de fucosa unido a GlcNac no impide la unión al receptor. Un análisis comparativo de la secuencia de seis de los aislados del virus del tipo H13 y otros subtipos indicó la presencia de diferencias sustanciales en la secuencia de aminoácidos en esta región en los virus del tipo H13.

Key words: avian influenza, ducks, gulls, hemagglutinin, receptor specificity

Abbreviations: 3'SL = Neu5Ac α (2-3)Gal β (1-4)Glc β ; 3'SLN = Neu5Ac α (2-3)Gal β (1-4)GlcNAc β ; HA = hemagglutinin; Neu5Ac α = alpha *N*-acetylneuraminic acid; PAA = polyacrylamide; RBS = receptor binding site; SLe^x = [Neu5Ac α (2-3)Gal β (1-4)][-Fuc α (1-3)]GlcNAc β ; SLe^a = [Neu5Ac α (2-3)Gal β (1-3)][-Fuc α (1-4)]GlcNAc β

The prototype variant of H13 influenza viruses A/gull/Maryland/704/1977 was isolated for the first time in the United States in 1977 (7). At the same time, H13 influenza viruses were obtained in the U.S.S.R. and could be routinely isolated among birds in the Family *Laridae* in the Volga River delta and in the North Caspian Region (13). In 1986, viruses of the same subtype were isolated from a mute swan in Japan, and additional isolations of this subtype virus were made in the Far-Eastern Region and the Black Sea Reserve (10,14). Single isolates of this subtype were also made from the birds of the *Anatidae* family (teal, coot, garganey). Isolations, primarily from the *Laridae* family of birds, the inability of these viruses to replicate in ducks (experimental data), and the substantial differences of HA and NP sequence from other subtypes served as the basis of a hypothesis of unique and restricted host range of these viruses (8).

The subsequent isolation of H13 influenza viruses from whales and the significant differences in the structure of the genes of viruses isolated in both hemispheres (2,12) increased the interest in these viruses.

This research deals with a more detailed comparative study of the receptor binding site (RBS) of the hemagglutinin (HA) protein of a number of H13 viruses isolated from birds from the family *Laridae* and other viruses obtained from birds from the family *Anatidae*.

MATERIALS AND METHODS

Viruses. The avian viruses were obtained from the virus repository of the D.I. Ivanovsky Institute of Virology in Moscow. These viruses were propagated in 9-to-10-day-old embryonated chicken eggs. For the

studies on virus binding, virus-containing allantoic fluids were centrifuged and used without further purification. In same experiments, the viruses were partially purified by 30% sucrose centrifugation.

Free *N*-acetylneuraminic acid (Neu5Ac), 3'-sialyllactose (3'SL, Neu5Ac α 2-3Gal β 1-4Glc) were purchased from Serva (Switzerland). Sialylglycopolymers bearing 3'-sialyl(*N*-acetylglucosamine) (3'SLN-PAA), [Neu5Ac α (2-3)Gal β (1-4)][-Fuc α (1-3)]GlcNAc β (SLe^x-PAA), [Neu5Ac α (2-3)Gal β (1-3)]-Fuc α (1-4)]GlcNAc β (SLe^a-PAA) moieties attached to the polyacrylamide carrier were synthesized as described (1,4).

The affinity of the influenza viruses for soluble receptor analogs was evaluated in a competitive assay based on the inhibition of the binding by the solid-phase immobilized virus of the standard preparation of bovine fetuin labeled with horseradish peroxidase (4). The data were expressed in terms of binding constants (Kass) formally equivalent to the association constants of virus/receptor analog complexes. For the calculation of the constants, concentration of the sialic acid residues in the solution was used both for the monovalent sialosides and for the polyvalent sialylglycopolymers. Molecular cloning and sequencing of influenza virus genes was performed as describe in Yamnikova *et al.* (11).

RESULTS AND DISCUSSION

We demonstrated earlier that the RBS of avian influenza viruses, regardless of the HA subtype, is of high conservation and possesses a high affinity to 3'-sialyllactose and other Neu5Ac(2-3)Gal-terminated oligosaccharides. The receptor determinant of most subtypes of avian influenza viruses is 3'-sialyllactose, the galactose moiety considerably contributes in binding, which is the reason affinity of viruses to sialyllactose is significantly higher than

Table 1. Binding of soluble receptor analogs by avian influenza viruses of different hosts.^A

Viruses		K _d (μM) to receptor analogs				
		Neu5Acα	3'SL	3'SLN-PAA	SLe ^x -PAA	SLe ^a -PAA
A/duck/France/46/82	H1N1	>2000	100	15	50	5
A/pintail/Primorie/695/76	H2N3	>2000	200	50	100	0.2
A/duck/Buryat/652/88	H3N8	1000	100	50	>100	20
A/duck/Czechosl/56	H4N6	>2000	300	10	>100	1
A/duck/Buryatia/1943	H4N6	>2000	300	10	50	2
A/duck/Pensilvania/10218/84	H5N3	2000	200	20	50	8
A/gull/Merilend/704/77	H13N6	1000	500	15	15	10
A/A/gull/Astrakhan/1421/74	H13N6	1000	800	40	10	10
A/gull/Astrakhan/78/81	H13N2	600	600	50	50	50
A/gull/Astrakhan/227/82	H13N6	1000	500	10	10	>50
A/gull/Astrakhan/75/83	H13N2	300	200	50	50	50
A/gull/Astrakhan/10/88	H13N6	2000	800	3	2	2
A/gull/Astrakhan/44/88	H13N6	2000	1000	30	20	30
A/gull/Astrakhan/1808/98	H13N6	1000	800	20	20	30
A/gull/Astrakhan/1846/98	H13N6	1000	500	10	4	2
A/Whale/Maine/328H/84	H13N6	3000	1000	10	10	10

^AThe dissociation constants (μM Neu5Ac) of virus complexes with soluble receptor analogs were determined in a competitive binding assay; lower values of K reflect stronger binding to the receptor. Values given in the table are obtained by averaging the data of three experiments and are approximated to one or two significant digits in accordance with the confidence interval.

to free Neu5Acα (5). However, this property is less pronounced in a number of H13 influenza viruses (9). The substitution G→S 228, typical of H2N2 and H3N2 human influenza viruses, is revealed in the RBS of H13 HAs. Even though this substitution does not dramatically change the specificity of the virus, it significantly transforms the receptor-binding phenotype characteristic of the influenza viruses isolated from ducks. For a detailed comparison of receptor activity of H13 HA viruses and typical isolates from ducks, affinity of all viruses to *N*-acetylneuraminic acid (Neu5Acα), 3'sialyllactose (3'SL) and to sialylglycopolymers bearing 3'-sialyl(*N*-acetylglucosamine) (3'SLN-PAA), [Neu5Acα(2-3)Galβ(1-4)][-Fucα(1-3)]GlcNAcβ (SLe^x-PAA) and [Neu5Acα(2-3)Galβ(1-3)][-Fucα(1-4)]GlcNAcβ (SLe^a-PAA) was determined. The last three polymer glycoconjugates were synthesized, and the contribution of carbohydrate residues linked to the galactose moiety in the binding with the receptor was determined.

As shown in Table 1, the difference in affinity between 3'SL and Neu5Acα in all studied H13 viruses is small, which indicates a less significant role of the galactose link in the binding with the receptor. The results of virus binding with polymer sialylglycoconjugates indicate that the method of linking the third moiety and the presence of an extra fucose

substitute in it influence the binding considerably. For viruses isolated from ducks, the suitable polymer is SLe^a-PAA (i.e., for them, a 1-3 binding between galactose and glucosamine is optimal). This finding is in accord with the reports on the effective recognition of gangliosides by the duck viruses (9). Fucose linked to the C-4 of glucosamine in SLe^a-PAA does not prevent the binding. However, in the case of a 1-4 linkage between galactose and glucosamine, the presence of fucose at the C-3 of glucosamine prevents the binding. For this reason, the affinity to SLe^x-PAA is lower than to SLN-PAA. H13 viruses isolated from the gulls differ in terms of their ability to interact with PAA sialylglycoconjugates. The affinity to all three polymers is uniform, and the presence of fucose at the C-3 of glucosamine does not prevent the binding.

The dissociation constants (mkM Neu5Ac) of virus complexes with soluble receptor analogs were determined in a competitive binding assay; lower values of K-diss reflect stronger binding to the receptor.

The data in Table 1 represents the average of three experiments, and these experiments are approximated to the one or two significant digits in accordance with the confidence interval. The results make it possible to suggest that such high differences in the receptor properties of viruses are

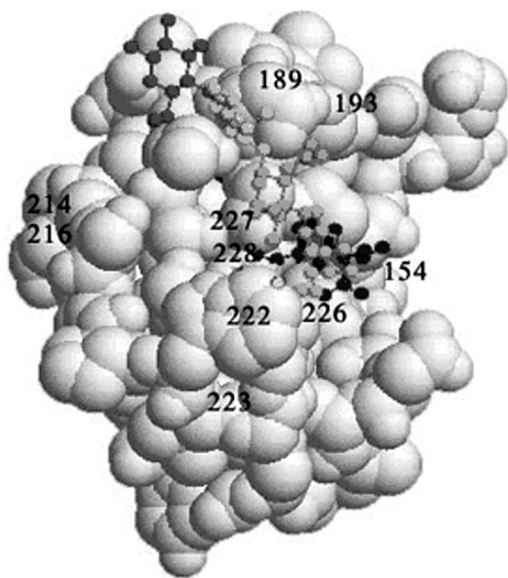


Fig. 1. Amino acids in RBS of HA H13 influenza viruses isolated from gulls, differing from duck viruses. Substitution is shown on the model of M. B. Eisen, *et al.* (3).

determined by the structure of their RBS. To support this assumption, we studied the primary structure of some H13 viruses isolated at different times in the gull colony in the Caspian Sea Basin and analyzed amino acid sequences of the RBS from GenBank or from the literature cited in this article. According to X-ray crystallography data analysis (3) of the human H3 HA complex of A/Aichi/68 influenza with LSTa—pentasaccharide, terminated with (2-3) sialic acid, the stalk of 3'-sialosides is directed into the left-hand upper corner of the RBS, between the 187-192 and 215-231 regions (Fig. 1).

The structural model of the H3 HA complexed with natural pentasaccharide LSTa (3) predicts that the asialic parts of Neu5Ac- α -(2-3)Gal—containing sialyloligosaccharides would protrude towards the left side of the HA receptor-binding pocket (Fig. 1). It seems likely, therefore, that duck influenza viruses

bind weakly to SLe^x-containing polymer because of sterical interference of fucose with the left side of the RBS (Fig. 1).

An additional fucose moiety, apparently, should be directed to the left, toward amino acids in positions 227, 219, 220, and 222, provided that the receptors are SLe^x and/or SLe^a. In this area, many amino acids are constant for all or practically all influenza virus subtypes. There are also many positions in which amino acids are not constant but coincide in such parameters as hydrophobicity, size, or charge.

A comparative analysis of six sequenced H13 viruses with other subtype viruses showed the presence of substantial differences in the composition of amino acids of this region in the H13 viruses. It appears from Table 2 that amino acids in positions 215, 228, and 229, constant for all subtypes, are replaced. Substitution of Pro→Leu in position 215 is capable of changing dramatically the loop configuration. Amino acids in positions 228 and 229 help shape the RBS. Amino acids in positions 228 and 226 are key amino acids responsible for recognition of 2-3 or 2-6 sialylgalactose receptors. In position 222, in all viruses isolated from ducks, there is a bulky amino acid, whereas in HA H13, it is substituted by a small amino acid, Gly. Small amino acids Ala, Gly, or Ser are also located in isolates from ducks in position 227; in the gulls, Arg or Lys is found. Substitutions are also found at position 231. A negatively charged amino acid is most often found in the HA of viruses isolated from ducks, whereas in the H13 HA, there is a positively charged Lys. Besides this radical reconstruction of the loop 215-231, there occurs a substitution of a bulky amino acid for a small one in position 191. Phe is found in position 154 instead of the dominant Lys found in other subtypes. All the aforesaid substitutions result in a transformation of the receptor phenotype of H13 HA viruses isolated from gulls. Results presented in Table 1 show that a receptor epitope of duck influenza viruses is not merely a sialic acid, and not

Table 2. Substituted amino acids in HA of H13 viruses.

Amino acid No. (H3 HA)	154	191	215	222	223	227	228	229	231
Duck virus— all subtypes	L, I ^A	Q, h	P	K, P, R, L, Q, W	V, I, a	A, G, S	G	R	D, E, n, t, s
H13 viruses (gull)	F	T, A	L	G	Y	R, K	S	W	K

^AInfrequent substitutions in HA of viruses isolated from ducks are denoted by a lowercase letter.

even sialylgalactose, but rather is a more complicated determinant, including the monosaccharide moiety. For H13 viruses, on the contrary, the role of the second monosaccharide seems to be reduced considerably, whereas the penultimate residues do not participate in the binding at all. Earlier, we showed that epithelial cells of the intestinal tract of chickens and ducks have different compositions of receptors for influenza viruses (6). It is possible that the differences are present in order to facilitate recognition of receptor determinants of the SLe^x type, with which duck viruses do not bind well, or to expand the set of potential receptors. As a result, H13 viruses have an advantage at the first stage of infection. This agrees with our long-term observations regarding the dominant circulation of H13 viruses in the colony of the gulls.

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ACKNOWLEDGMENTS

This work was supported by research grant ISTC 1881p and portions of research grants 99-04-48064 and 01-04-49300 from the Russian Foundation for Basic Research.